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(54) Title: METHOD FOR ABSOLUTE COUNTING OF RARE CELLS

(57) Abstract

A method for absolute counting of rare cells (i.e., < 5 %) in a sample of cells is described. The method utilizes flow cytometry and a reagent composition comprising fluorescent beads, one or more fluorescent markers that differentially react with cell populations in the sample and one or more fluorescent markers that selectively react with the rare cells. The method has particular utility in counting progenitor cells.

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METHOD FOR ABSOLUTE COUNTING OF RARE CELLS

This application is a continuation-in-part of co-pending and commonly assigned U.S. Serial No. 08/046,343, filed 8 April 1993, which is a continuation of U.S. Serial No. 07/570,569, filed 7 August 1990 (now abandoned).

Field of the Invention

This invention relates to a method for determining the absolute number of rare cells in a sample of cells, and more particularly relates to a method for determining the absolute number of progenitor cells in a sample of peripheral blood or bone marrow cells by means of flow cytometry.

Background of the Invention

Traditionally, in immunofluorescence flow cytometric measurements, cell populations are quantified by their relative distributions (*i.e.*, as a percentage of cells in the sample counted). In peripheral blood, leukocytes are out-numbered by red blood cells approximately 1000 to 1. Thus, most work involving flow cytometry has involved the lysis of red blood cells followed by centrifugation, resuspension, labelling and counting of the remaining cells. Unfortunately, this process destroys the ability to accurately count the remaining cells as a function of blood volume.

With the advent of the AIDS crisis, $CD4^+$ cell counting became more important in the diagnosis and monitoring of the disease. In one method to obtain accurate measurements, absolute CD4 counts were attempted by multiplying a leukocyte count by the percentage of lymphocytes (both as determined by a hematology analyzer (*e.g.*, Coulter Counter)

with the percentage of $CD4^+$ as determined by flow cytometry. *See* NCCLS Guidelines Report. This approach suffers from large imprecision due to well recognized variations in precision between hematology instruments.

Other approaches involve the use of a syringe delivery system coupled to a flow cytometer where a known sample volume is delivered to the instrument and the number of events in that sample volume is counted. Still other approaches involve the addition of a known number of fluorescent beads to a known sample volume and then counting the number of beads and events per unit of time. The ratio of beads to cells provides the number of cells per unit volume. *See* U.S. Pat. No. 4,110,604.

These approaches are appropriate for counting cells in populations that exceed 5% of the sample. For counting rare cells, however, these approaches may not achieve the desired accuracy. Antigen density and rare cell distribution in the sample make discrimination between "positive" and "negative" cells difficult when relying on a single marker for the rare event. Thus, Gross *et al.* developed a method for detecting very rare cells (*i.e.*, on the order of 1×10^{-6}) using a combination of markers. (*See* Cytometry, 14:519, 1993; *and see* U.S. Serial No. 08/009,185, filed 26 January 1993.) That method has particularly utility in the evaluation of minimum residual disease in breast cancer, for example.

In between very rare cells and cell populations that exceed 5%, there are cell populations that are "rare" for which absolute counting is becoming increasingly important. In U.S. Patent No. 4,714,680, Civin described a population of pluripotent lympho-hematopoietic cells which were substantially free of mature lymphoid and myeloid cells. Civin also described an antigen, MY-10 (also known as CD34) and a monoclonal antibody (of the same name) thereto, which was present on these cells. These "progenitor" cells make up to about 1% of all cells in normal adult bone marrow, and

generally comprise a mixture of common stem cells, hematopoietic stem cells and lineage committed progenitor cells with the latter cells predominating.

Civin described a number of therapeutic uses for progenitor cells. Many of these uses involve the transplantation of $CD34^+$ cells into the bone marrow of a patient having undergone ablative therapy for the purpose of not only reconstituting the patient's hematopoietic system but also avoiding graft versus host disease. Still other therapies involve the transplantation of $CD34^+$ cells into peripheral blood.

Therapies involving the transplantation of progenitor cells currently are being conducted by a number of companies around the world. In many of these methods, leukocytes are collected from peripheral blood, cord blood or bone marrow and the $CD34^+$ cells are separated from the remainder of the cells in the sample using either CD34 monoclonal antibodies in a positive selection step or in combination with one or more other antibodies to deplete non-progenitor cells. The cells may be harvested from an allogeneic source or from the patient. If obtained from the patient, the patient first may be given growth factors, such as GM-CSF or G-CSF, prior to harvesting in order to boost the number of circulating progenitor cells.

Means for performing the collection process include fluorescence activated cell sorting (*see, e.g.*, U.S. Pat. No.s 3,826,364 and 5,030,002), biotin/avidin columns (*see, e.g.*, U.S. Pat. No.s 5,215,927, 5,225,353, and 5,240,856) and magnetic bead based systems (*see, e.g.*, WO91/09141 and Kato *et al.*, Cytometry, 14:384 (1993)). Systems comprising flow cytometers, data gathering and analysis hardware and software, and reagents are sold by a number of commercial entities including Becton Dickinson Immunocytometry Systems ("BDIS").

In addition to therapies where progenitor cell populations are used, there has been additional work on subsets of progenitor cells. (These cells are even rarer than progenitor cells in most cell samples.) In this work, Terstappen and others focused on functional studies of CD34 subsets of cells in order to find populations of cells enriched for pluripotent hematopoietic stem cells and totipotent common stem cells. *See* U.S. Serial No. 07/895,491, filed 8 June 1992, which is a continuation-in-part of U.S. Serial No. 07/759,092, filed 6 September 1991. In an article published in Scientific American in December 1991, Golde generically described stem cells and described research into isolating stem cells and methods of using stem cells for therapeutic uses.

In all cases, it is important to know how many $CD34^+$ cells are present within the material to be transplanted. Generally, it is believed that 1×10^6 $CD34^+$ cells per kilogram of body weight are necessary in any donor material to have a reasonable likelihood of successful engraftment. In other cases (*i.e.*, where cytokines are used to stimulate progenitor cell production prior to harvesting), it is important to know the level of progenitor cells in the blood so as to time the harvest of those cells from the donor with maximum production. Thus, it is important to be able to accurately measure the absolute number of progenitor cells (or cells in subsets thereof). Flow cytometry provides a convenient means for rapidly and accurately counting cells.

Summary of the Invention

The invention comprises reagents and methods for accurately determining the absolute numbers of rare cells (*i.e.*, <5%) in a sample. The method has particular utility in determining the number of $CD34^+$ cells in a sample and subsets of $CD34^+$ cells such as $CD34^+/CD38^-/HLA\text{-}DR^+$ cells.

The method comprises the steps of: a) labelling cells in a sample with a composition comprising 1) one or more fluorescent markers that react differentially react with all of the cells in the sample, 2) one or more fluorescent markers that selectively react with the rare cells, and 3) a known number of fluorescent beads, wherein all of the fluorescent markers have emission spectra that are distinguishable; and

b) analyzing the labelled cells by means of flow cytometry comprising the steps of 1) setting a fluorescence threshold on the fluorescence emitted by the cells labelled with the marker of a)1) so as to include all labelled cells and beads in a data analysis set, 2) analyzing the cells in the set that meet or exceed the threshold for one or more of light scatter and fluorescence emissions, 3) using the data recorded to discriminate between and among the various cell populations in the sample and beads, 4) counting the number of beads and rare cells and determining the ratio thereof.

In a preferred embodiment of the invention, the method comprises the steps of: a) labelling cells in a sample with a composition comprising 1) one or more monoclonal antibodies, labelled with a first fluorochrome, that differentially react with all cell populations in the sample, 2) one or more monoclonal antibodies, labelled with a fluorochrome that has an emission spectra that is distinguishable from the first fluorochrome, that selectively react with rare cells, and 3) a known number of fluorescent beads; and

b) analyzing the labelled cells by means of flow cytometry comprising the steps of 1) setting a fluorescence threshold on the fluorescence emitted by the cells labelled with the fluorescent dye of a)1) so as to include all labelled cells and beads in a data analysis set, 2) analyzing the cells in the set that meet or exceed the threshold for one or more of light scatter and fluorescence

emissions, 3) using the data recorded to discriminate between and among the various cell populations in the sample and beads, 4) counting the number of beads and rare cells and determining the ratio thereof.

In this method, the composition may be added to the analysis tube before or after the cell sample is added. Alternatively, each of the elements of the composition may be added separately or in sub-combinations. It is preferred to provide the elements as a unitary composition.

The beads used in the practice of this invention should have certain properties. First, they should be small (*i.e.*, between 0.2μm and 20μm, 2μm are preferred) so as to stay suspended in the mixture and not settle any faster than the cells in the sample. Second, they should be made of a material that avoids clumping or aggregation. Third, they should be fluorescent and/or of such size so as to be distinguishable from the cells in the sample. Fluorescence can be achieved by selecting the material that comprises the microparticle to be autofluorescent or it can be made fluorescent by being tagged with a fluorescent dye.

If fluorescence is used, the fluorescence of the beads must be such that in one fluorescence channel it is sufficiently greater than noise from background so as to be distinguishable and also must be distinguishable in other fluorescence channel(s) from the fluorescent dye(s) used as part of the fluorescent marker(s). One log difference between the dye(s) and the bead fluorescence is sufficient. Beads having these properties may be selected from the group consisting of fixed chicken red blood cells, coumarin beads, liposomes containing a fluorescent dye, fluorescein beads, rhodamine beads, fixed fluorescent cells, fluorescent cell nuclei, microorganisms and other polymeric beads tagged with a fluorescent dye. Beads of the latter type are commercially available from Molecular Probes, Inc. and Flow Cytometry Standards Corp.

Fluorescent markers may comprise fluorescent dyes, such as nucleic acid dyes, and/or monoclonal antibodies (or other

reagents that have a high binding affinity for a particular target molecule) that are labelled with a fluorochrome.

Fluorochromes for monoclonal antibodies may be selected from the group consisting of fluorescein isothiocyanate ("FITC"), phycoerythrins ("PE"), allophycocyanin ("APC"), peridinin chlorophyll complex protein ("PerCP", BDIS), CY3 and CY5 (Biological Detection Systems, Inc.), Texas Red (Molecular Probes, Inc.) and tandem conjugates thereof, such as PE/CY5. *See* U.S. Pat. No.s 4,542,104 and 4,520,110.

Fluorescent dyes that exhibit a Stokes shift when reacted with nucleic acids include Thiazole Orange (BDIS), 7-amino-actinomycin D (Sigma), SY-III-8 (Molecular Probes, Inc.), LDS-751 (Molecular Probes, Inc.), and dyes described in U.S. Pat. No.s 4,544,546, 4,945,171 and 5,066,580.

It is understood that the fluorescence of the beads, if any, and fluorescent markers should be such that each of their peak emission spectra are distinguishable, preferably without compensation.

Means for discriminating between the cell populations and beads in the sample comprise both cell analyzers (*e.g.*, FACScan brand flow cytometer, BDIS) and cell sorters (*e.g.*, FACSort brand flow cytometer, BDIS). A threshold is set according to manufacturer's directions for fluorescence emitted from one of the fluorescent markers. This may be a nucleic acid dye or a fluorochrome used in conjunction with a monoclonal antibody to differentially label all of the cells. (In an alternative embodiment, multiple thesholds may be set in an "A and B" or "A but not B" configuration.) For each event that exceeds the fluorescence threshold(s), one or more light scatter and fluorescence emission parameters are recorded. (*See* U.S. Pat. No. 4,727,020.) It is preferred that two light scatter and three fluorescence parameters are recorded and used to discriminate between and among cell populations and beads. The number of cells in at least the rare cell population and the number of beads then

is determined, and a ratio prepared yielding the absolute number of cells. It is understood that additional populations of cells (*e.g.*, leukocytes or T lymphocytes) also may be counted so long as they are identified by one or more markers or other parameters (*e.g.*, $CD45^+$ or $CD3^+$).

In analyzing the data to be gathered using this method, it desirable to be sure that the instrument on which the sample will be analyzed has been set up properly and that the sample has been properly prepared (*e.g.*, all of the reagents have been added).

Samples of cells can be obtained from any tissue source, such sources included peripheral blood, cord blood, bone marrow, thymus, spleen or lymph node. Sources of cells from peripheral blood and bone marrow are particularly applicable to the methods of this invention.

In another embodiment, the composition may be modified to replace the fluorescently labelled antibody specific for the rare cells with a fluorescently labelled irrelevant isotpye control. The sample may be split into aliquots with one being stained with the isotype composition and the other with the standard composition. The former aliquot then can be analyzed first to determine background fluorescence levels prior to analysis with the second aliquot.

The method has particular utility in counting $CD34^+$ cells (and subsets thereof) from peripheral blood, cord blood or bone marrow. In this mode, the method comprises the steps of: a) labelling cells from a sample with a composition comprising 1) a nucleic acid dye that will selectively react with nucleated cells, 2) a monoclonal antibody labelled with a first fluorochrome that differentially reacts with mature lymphoid, neutrophil, erythroid and monocytic cells and weakly with progenitor cells, 3) a CD34 antibody labelled with a second fluorochrome, and 4) a known number of fluorescent beads; and

b) analyzing the labelled cells by means of flow cytometry comprising the steps of 1) setting a fluorescence threshold on the fluorescence emitted by the nucleic acid dye so as to include all nucleated cells and beads, 2) analyzing the cells and beads in the sample that meet or exceed the threshold for light scatter and fluorescence emissions, 3) using fluorescence emissions and scatter data recorded to discriminate between and among the various cell populations in the sample and beads, 4) counting the number of beads and $CD34^+$ cells and determining the ratio thereof.

In this method, Thiazole Orange and/or SY-III-8 are preferred nucleic acid dyes. SY-III-8 is especially preferred. CD45 is a preferred antibody for differentially reacting with all leukocytes. A tandem conjugate of PE/CY5 is preferred as one fluorochrome and PE is preferred as the other fluorochrome. Nile Red beads (Molecular Probes, Inc.) are preferred.

In an alternative embodiment, the nucleic acid dye may be eliminated and the threshold set on the fluorescence emitted by the cells labelled with the first fluorochrome.

Other rare cell populations that may be counted by this method include antigen specific B or T lymphocytes and specific effector cells (*e.g.*, cytotoxic T cells).

Description of the Figures

FIG. 1 comprises two dot plots of lysed normal peripheral blood to which 50,000 fluorescent beads were added and then analyzed by means of flow cytometry wherein 1A is a plot of transformed orthogonal light scatter versus forward light scatter and 1B is a plot of log fluorescence emissions at 564-606 nm versus log fluorescence emissions at 515-545 nm.

FIG. 2 comprises two dot plots of lysed normal peripheral blood treated as in FIG. 1 to which the nucleic

acid dye SY-III-8 also was added wherein 2A is a plot of transformed orthogonal light scatter versus log fluorescence for events collected using a light scatter threshold and 2B is a plot of transformed orthogonal light scatter versus log fluorescence for events collected using a fluorescence threshold.

FIG. 3 comprises two dot plots of lysed abnormal peripheral blood treated as in FIG. 2 to which the CD45 PE/CY5 also was added for events collected using a fluorescence threshold wherein 3A is a plot of log SY-III-8 fluorescence versus forward light scatter and 3B is a plot of transformed orthogonal light scatter versus log PE/CY5 fluorescence.

FIG. 4 comprises a series of six dot plots of lysed normal peripheral blood treated as in FIG. 3 for events collected using a fluorescence threshold to which either an IgG_1 PE control antibody also was added (3A, 3C and 3E) or a CD34 PE antibody was added (3B, 3D and 3F) wherein 3A and #B are plots of log PE fluorescence versus log SY-III-8 fluorescence, 3C and 3D are plots of transformed orthogonal light scatter versus forward light scatter and 3E and 3F are plots of transformed orthogonal light scatter versus log PE/CY5 fluorescence.

FIG. 5 comprises histograms showing the number of $CD34^+$ cells per µl in 5 replicate analyses of two different lysed peripheral blood samples ("low" CD34 counts closed box, "high" CD34 counts open box) treated as in FIG. 4 with a CD34 PE antibody for events collected using a fluorescence threshold wherein 5A represents one sample treated once and analyzed five times and 5B represents one sample split into five aliquots each aliquot treated separately and then analyzed.

FIG. 6 comprises a series of eight dot plots of lysed leukopheresis samples to which have been added 50,000 fluorescent beads, SY-III-8, CD45 PE/CY5 and IgG_1 PE (6A-6D)

or 50,000 fluorescent beads ,SY-III-8, CD45 PE/CY5 and CD34 PE (6E-6H) wherein 6A and 6E are plots of transformed orthogonal light scatter versus forward scatter, 6B and 6F are plots of transformed orthogonal light scatter versus log PE/CY5 fluorescence, 6C and 6G are plots of log PE versus log SY-III-8 fluorescence, and 6D and 6H are plots of log PE versus log PE/CY5 fluorescence.

Detailed Description

Bone marrow aspirates, peripheral blood and leukopheresis samples were collected from patients and normal donors. Heparin was used as an anticoagulant for the bone marrow samples and $EDTA_{K3}$ as an anticoagulant for peripheral blood samples. For each test, 50µl of peripheral blood, bone marrow or leukopheresis sample was used.

The samples were incubated with 10µl of control or test reagent and 50,000 freeze-dried 2.49µm Nile Red beads. The control and test reagent mixture consisted of a nucleic acid dye (*i.e.*, 5ng SY-III-8, Molecular Probes, Inc.), a PE/CY5 tandem conjugate of C45 (100ng clone HLe-1, BDIS) and a PE conjugate of CD34 (100ng clone HPCA-2, BDIS) in the test reagent or IgG_1 control antibody (50ng, BDIS) in the control reagent. In some experiments, a mixture of Thiazole Orange and SY-III-8 was used. Samples were incubated for 20 minutes at room temperature. After incubation, the sample was diluted with 0.5ml of a red cell lysing solution (FACSLysing solution, BDIS).

SY-III-8 is a nucleic acid dye with a high preference for DNA over RNA. Thiazole Orange is a similar type dye and also has a preference for DNA over RNA but not to the same degree. The use of Thiazole Orange to label nucleated cells is described in U.S. Pat. No. 4,883,867. SY-III-8 is excitable at 488nm and its emission spectra is distinguishable from that of PE/CY5 and PE.

Flow cytometric analysis was performed on a FACScan brand flow cytometer (BDIS) equipped with an Argon laser (488nm). Data acquisition was performed with LYSIS II brand data acquisition software (BDIS). Forward light scatter, orthogonal light scatter and three fluorescence signals were determined for each cells, and sorted in listmode data files. Each peripheral blood measurement consisted of 20,000 listmode events containing all events that exceeded a threshold set on SY-III-8 fluorescence (i.e., on nucleated cells). The analysis of the listmode data files was performed with PAINT-A-GATE brand data analysis software (BDIS). (*See* U.S. Pat. No. 4,845,653.) Orthogonal light scatter data was transformed by the method disclosed in U.S. Pat. No. 5,224,058.

To illustrate the one aspect of the current state of the art, 50,000 fluorescent beads were added to a sample of peripheral blood prepared as described above. An event threshold was set on orthogonal light scatter and data collected for 20,000 events on a flow cytometer as described above. FIG. 1 shows the light scatter and fluorescence properties of the beads. The beads are depicted red, the cells green and debris gray. The data was analyzed and showed that 9.8% of the events were beads and 28.6% of the events were cells. Knowing that 50,000 beads were added to 50µl of blood, the absolute number of cells is calculate to be 2.9×10^3 per µl. Because of the poor separation between debris and cells at the light scatter threshold, however, this number may not be accurate and, as importantly, may vary from sample to sample or instrument to instrument. Thus, merely adding fluorescent beads to a sample does not necessarily result in accurate absolute counting.

To improve the discrimination between cells and debris, a nucleic acid dye may be added to the sample preparation. (*See* U.S. Pat. No. 5,057,413.) Using the combination of

light scatter and nucleic acid content adds an additional dimension over the method disclosed in FIG. 1.

Referring to FIG. 2, a peripheral blood sample was prepared as in FIG. 1; however, the nucleic acid dye SY-III-8 was added. FIG. 2A shows a plot of orthogonal light scatter versus log fluorescence with the threshold set on orthogonal light scatter. Debris, cells and beads are colored as in FIG. 1. Data analysis of the sample revealed that beads accounted for 9.6% of the events, nucleated cells 34.1% and non-nucleated debris 56.3%.

Setting the event threshold on nucleic acid dye fluorescence gives a very different result. FIG. 2B shows that the percentage of beads was 22.8% while the percentage of nucleated cells was 77.0% of the events measured. Using the approach in FIG. 2A, the absolute number of nucleated cells was determined to be 3.55×10^3 per µl whereas in FIG. 2B the absolute number of cells was determined to be 3.38×10^3 µl. The use of light scatter as the event threshold, therefore, introduced a 5% error.

For those cell populations to be measured that are not rare (*i.e.*, > 5%), current methods of immunofluorescence measurements using a nucleic acid dye, fluorescent beads and a single immunofluorescent marker for the cell population of interest is appropriate. (*See* U.S. Pat. No. 5,047,321.) For rare cell populations, however, the density of the cell surface antigen(s) becomes increasingly important in order to distinguish between cells that are "positive" or "negative" for the antigen(s) of interest. One problem that contributes to this difficulty is that monoclonal antibodies will generally have some level of non-specific binding. This is particularly troublesome for cells exhibiting the Fc receptor for which myeloid cells are the most notorious.

To increase the ability to identify rare cell populations, a fluorescently labelled monoclonal antibody that reacts differentially with all leukocytes outside the

cell population(s) of interest but not with the rare cell population(s). In this example, CD45 was fluorescently labelled because it reacts differentially with all leukocytes but is only dimly expressed on progenitor cells.

FIG. 3 illustrates a peripheral blood sample from a chemotherapy patient to which the composition used in FIG. 2 was added. CD45 PE/CY5 also was added to the composition. Beads were colored red, monocytes blue, lymphocytes green, neutrophils and eosinophils yellow and nucleated red cells purple. The cell population colored gray contains progenitor cells among which are $CD34^+$ cells, plasma cells and basophilic granulocytes. Data analysis for events exceeding the SY-III-8 threshold shows that in a plot of transformed orthogonal light scatter versus log PE/CY5 fluorescence, each of the cell populations falls into a defined region including progenitor cells. In this example, monocytes were 12.5% of the events (0.77×10^3 per µl), lymphocytes were 15.8% of the events (0.98×10^3 per µl), neutrophils and eosinophils were 50.2% of the events (3.1×10^3 per µl), nucleated red cells were 3.5% of the events (0.22×10^3 per µl), and progenitor cells were 1.75% of the events (0.11×10^3 per µl).

Although the use of the nucleic acid dye and CD45 alone appear sufficient to discriminate leukocytes from progenitor cells, to accurately enumerate $CD34^+$ cells, it is necessary to add a fluorescently labelled monoclonal antibody that is specific for CD34. MY-10, the antibody discovered by Curt Civin, was classified by the International Workshop on Human Leukocyte Antigens as falling with the cluster designated as "CD34." CD34 monoclonal antibodies are commercially available from a number of sources including BDIS.

Referring to FIG. 4, a sample of peripheral blood was split into two aliquots and treated as in FIG. 3; however, to one aliquot, a PE labelled IgG_1 isotype control was added to the composition while in the other aliquot a PE labelled CD34

antibody was added. FIG.s 4A, 4C and 4E show the control aliquot while FIG. 4B, 4D and 4F show the aliquot with CD34 PE. Cells and beads are colored as in FIG. 3 with $CD34^+$ cells colored black. In FIG. 4E, the arrow points to the expected location of the $CD34^+$ cells which is confirmed in FIG. 4F. $CD34^+$ cells from FIG. 4F comprise only about 30% of the progenitor cells identified in the space marked by the arrow in FIG. 4E.

In this example, no compensation between the three fluorescent labels was used. Compensation would lead to a subtraction of fluorescence signals of each of the labels from each of the three fluorescence channels in the flow cytometer. This decreases the density of events in each channel. Consequently, it is more difficult to define the area of interest containing the rare events.

To test the performance of CD34 absolute counting using the described method, two leukopheresis samples, one with relatively high and one with relatively low numbers of $CD34^+$ cells, were obtained. Referring to FIG 5A, each sample was treated as in FIG. 4 and then that sample was analyzed five consecutive times as in FIG. 4. Referring to FIG. 5B, blood from both leukopheresis samples further were split into five aliquots which then each were treated as in FIG.4 and analyzed.

Referring to FIG. 5A, the mean absolute number of $CD34^+$ cells from the "low" sample was 37.2 per µl (CV 10.4%) and the mean absolute number of cells from the "high" sample was 399.1 per µl (CV 8.2%). The mean percentage of $CD34^+$ cells from the "low" sample was 0.2% (CV 4.4%) and the mean percentage of cells from the "high" sample was 1.2% (CV 6.0%). The mean absolute number of nucleated cells in the "low" sample was 24.6×10^3 per µl (CV 4.7%), and the mean absolute number of nucleated cells in the" high" sample was 34.1×10^3 per µl (CV 3.8%).

Referring to FIG. 5B, the mean absolute number of $CD34^+$ cells from the "low" sample was 39.7 per µl (CV 9.7%), and the mean absolute number of cells from the "high" sample was 322.2 per µl (CV 9.0%). The mean percentage of $CD34^+$ cells from the

"low" sample was 0.2% (CV 13.3%) and the mean percentage of cells from the "high" sample was 1.1% (CV 9.3%). The mean absolute number of nucleated cells in the "low" sample was 26.7 x 10^3 per µl (CV 3.8%), and the mean absolute number of nucleated cells in the" high" sample was 29.8 x 10^3 per µl (CV 5.5%).

To demonstrate the utility of the method when the sample is split into aliquots and an isotype control is substituted in the composition, a suspension of beads first was used to set the fluorescence and scatter detectors of the instrument by placing the beads in a specific location and adjusting the detectors accordingly. The composition used in FIG. 4 (both IgG control and CD34 antibody) then were added to samples of lysed peripheral blood. Using information from the control sample, the fluorescence locations of $CD34^+$ cells and beads were determined. (*See* FIG.s 6A-6D.) The sample containing CD34 then was run and 40,000 events acquired. The cells in FIG.s 6F-6I meet all the criteria for $CD34^+$ cells.

It will be appreciated that this method makes use of monoclonal antibodies. Other markers that specifically react with antigens present on cells also may used. For example, Fab fragments, single chain binding proteins derived from monoclonal antibodies and nucleic acid binding proteins as described in WO91/19813 also may be used in this method. It is not critical that monoclonal antibodies be used. It is critical that the marker be selective for the desired antigen or molecule.

All publications and patent applications mentioned in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publication and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

It will be apparent to one of ordinary skill in the art that many changes and modifications can be made in the

invention without departing from the spirit or scope of the appended claims.

Claims

1. A method for counting the absolute number of progenitor cells in a sample comprising the steps of:

a) labelling cells in a sample with a composition comprising 1) a nucleic acid dye that will selectively react with nucleated cells, 2) a monoclonal antibody, labelled with a first fluorochrome, that will selectively react with mature lymphoid, neutrophil, erythroid and monocytic cells and weakly with progenitor cells, 3) a CD34 antibody labelled with a second fluorochrome, and 4) a known number of fluorescent beads; and

b) analyzing the labelled cells by means of flow cytometry comprising the steps of 1) setting a fluorescence threshold on the fluorescence emitted by the nucleic acid dye so as to include all nucleated cells and beads, 2) analyzing the cells and beads in the sample that meet or exceed the threshold for light scatter and fluorescence emissions, 3) using fluorescence emissions and scatter data recorded to discriminate between and among the various cell leukocyte populations in the sample and beads, 4) counting the number of beads and $CD34^+$ cells and determining the ratio thereof.

2. The method of claim 1 wherein the nucleic acid dye is SY-III-8.

3. The method of claim 1 wherein the monoclonal antibody is CD45.

4. The method of claim 3 wherein the first fluorochrome is PE/CY5.

5. The method of claim 1 wherein the CD34 monoclonal antibody is labelled with PE.

FIG - 1A

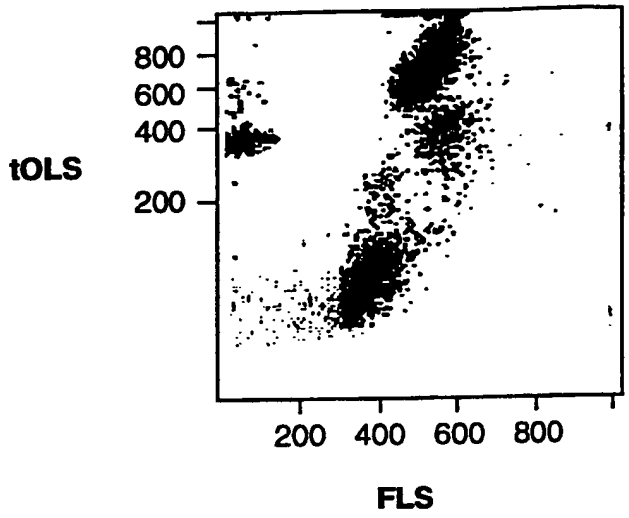

800
600
400
200
tOLS
200
400
600
800
FLS

FIG - 1B

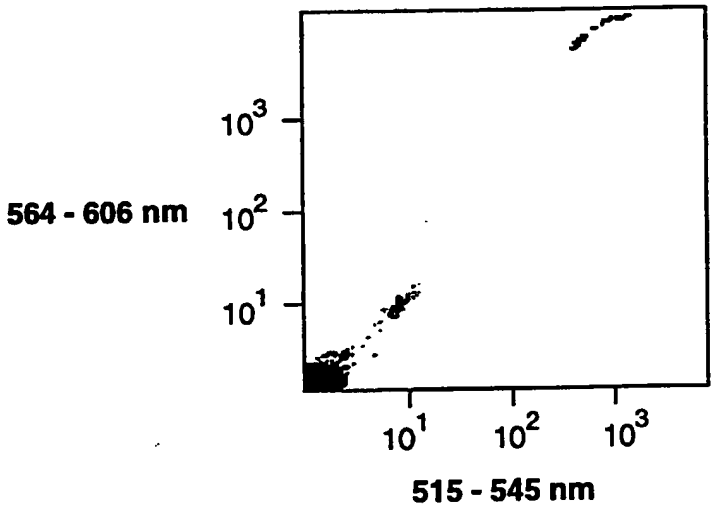

10^3
10^2
10^1
564 - 606 nm
10^1
10^2
10^3
515 - 545 nm

FIG - 2A

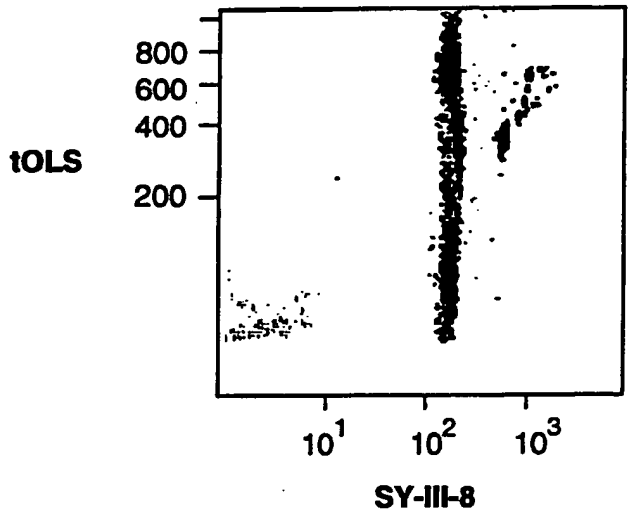

800
600
400
200
tOLS
10^1
10^2
10^3
SY-III-8

FIG - 2B

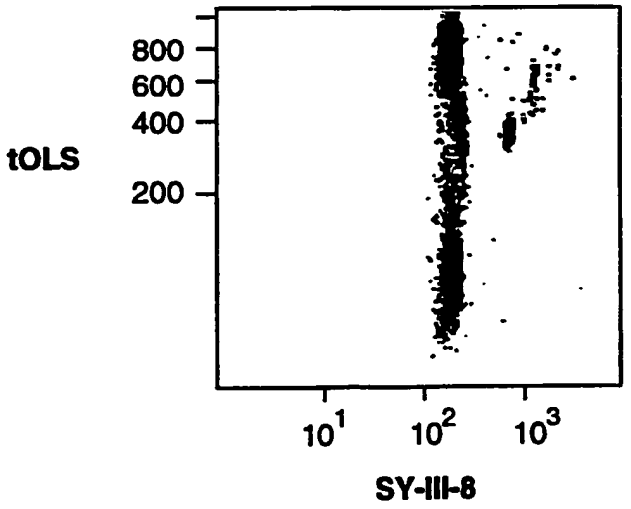

800
600
400
200
tOLS
10^1
10^2
10^3
SY-III-8

FIG - 3A

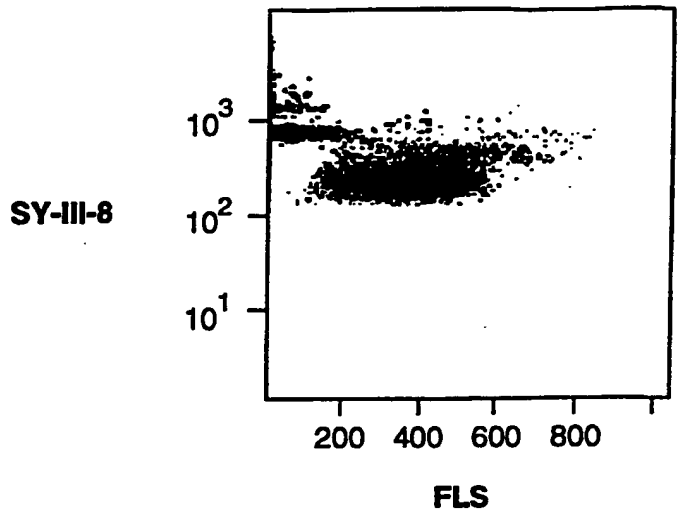

SY-III-8
10^3
10^2
10^1
200
400
600
800
FLS

FIG - 3B

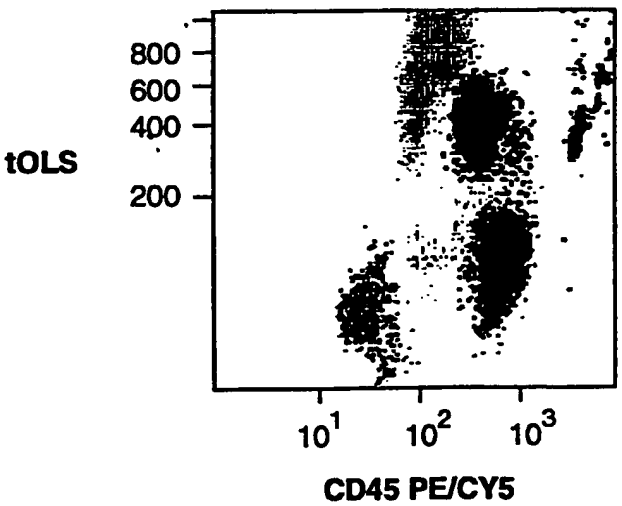

tOLS
800
600
400
200
10^1
10^2
10^3
CD45 PE/CY5

FIG - 4A

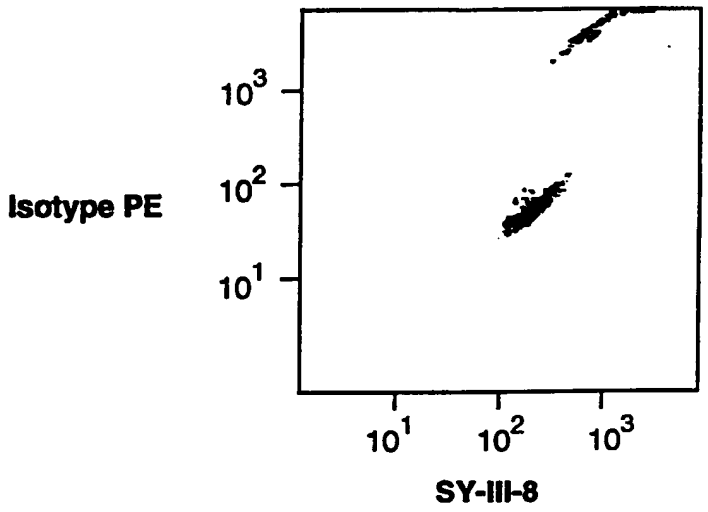

Isotype PE
10^3
10^2
10^1
10^1
10^2
10^3
SY-III-8

FIG - 4B

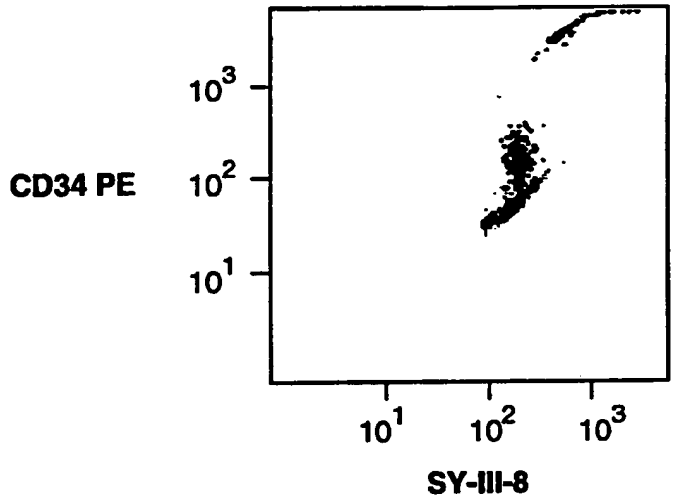

CD34 PE
10^3
10^2
10^1
10^1
10^2
10^3
SY-III-8

FIG - 4C

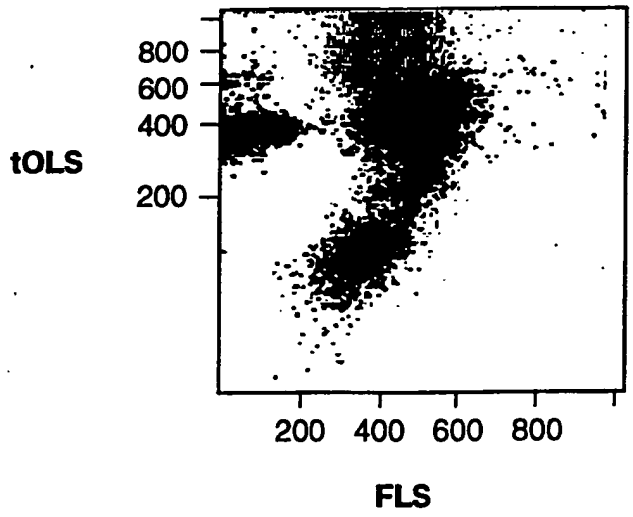

800
600
400
200
tOLS
200 400 600 800
FLS

FIG - 4D

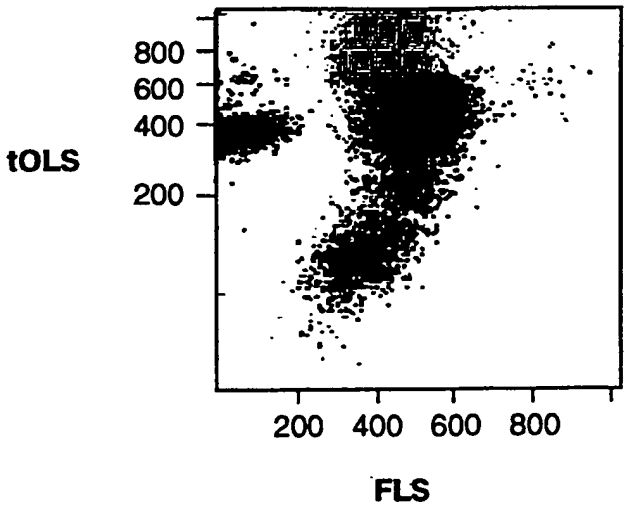

800
600
400
200
tOLS
200 400 600 800
FLS

FIG - 4E

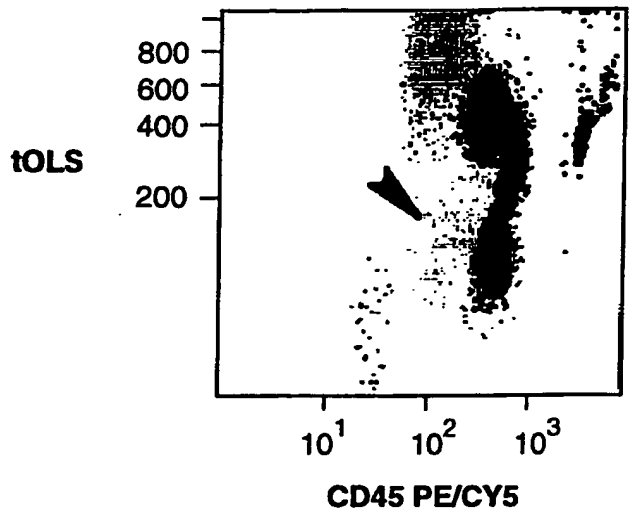

800
600
400
200
tOLS
10^1
10^2
10^3
CD45 PE/CY5

FIG - 4F

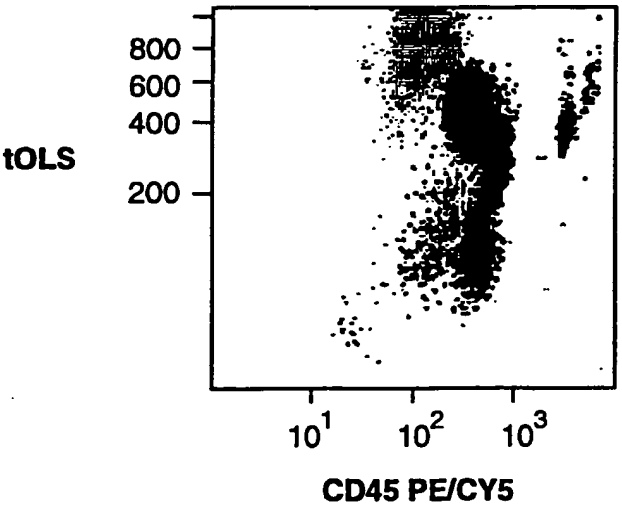

800
600
400
200
tOLS
10^1
10^2
10^3
CD45 PE/CY5

FIG - 5A

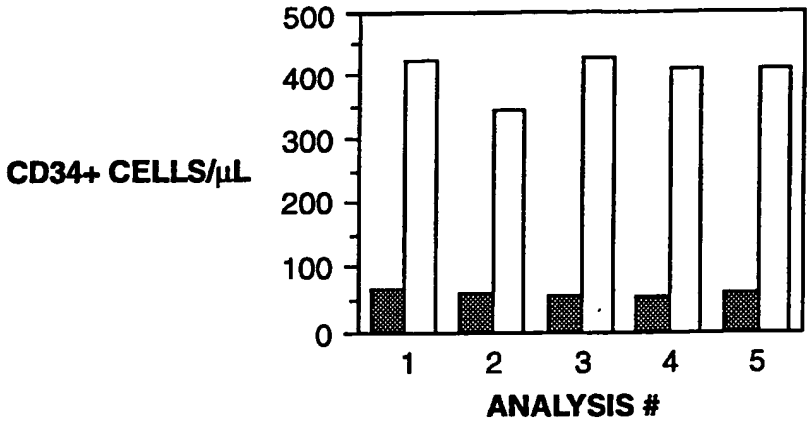

CD34+ CELLS/µL
500
400
300
200
100
0
1
2
3
4
5
ANALYSIS #

FIG - 5B

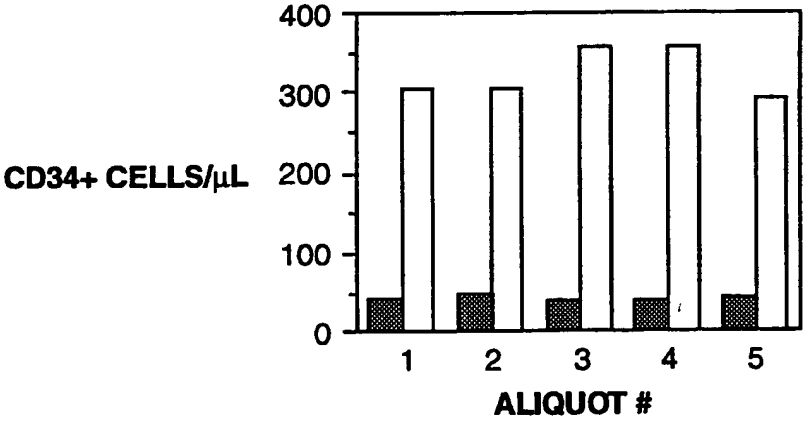

CD34+ CELLS/µL
400
300
200
100
0
1
2
3
4
5
ALIQUOT #

FIG - 6A

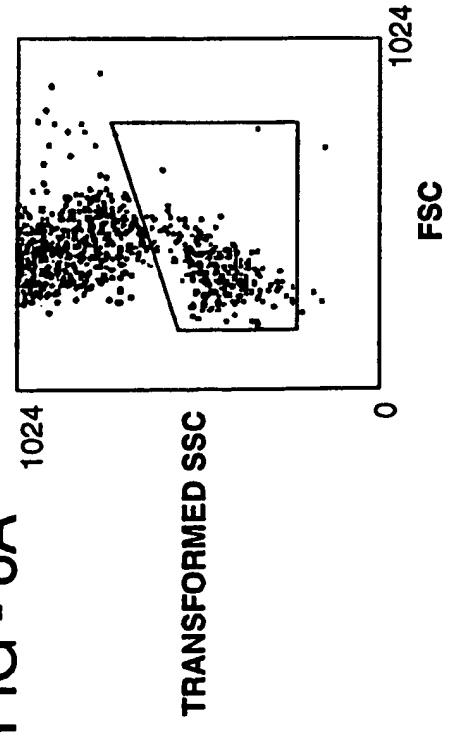


FIG - 6B

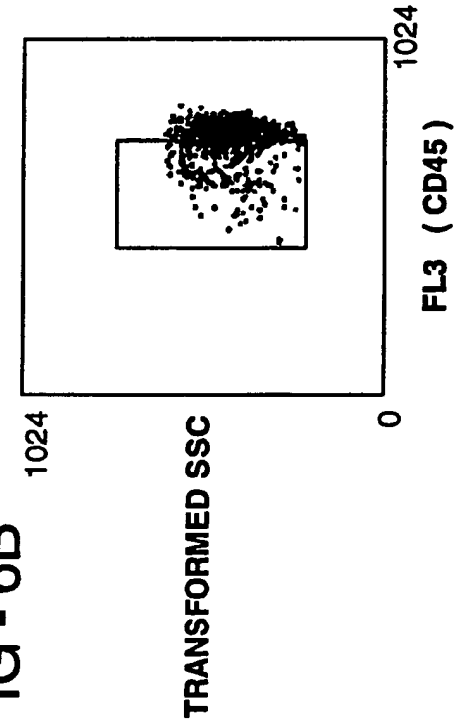


FIG - 6C

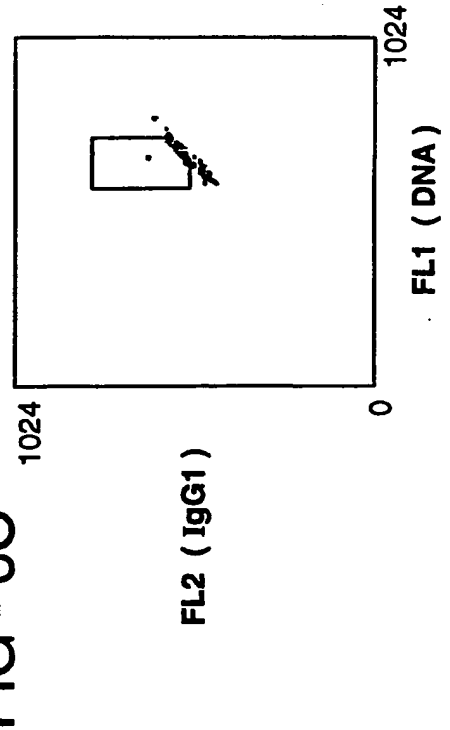
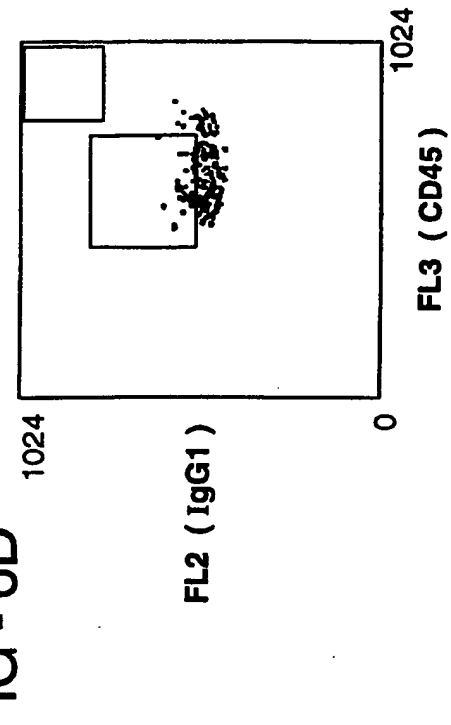


FIG - 6D



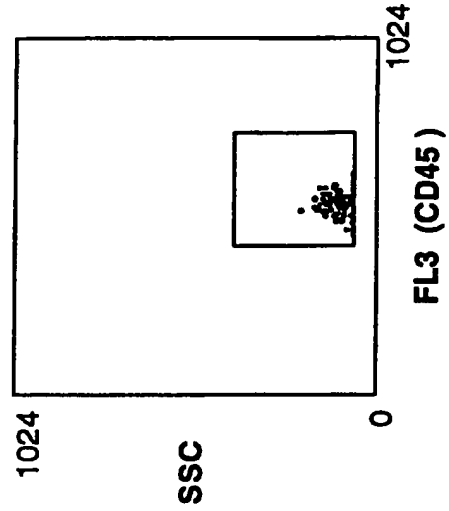


FIG - 6F

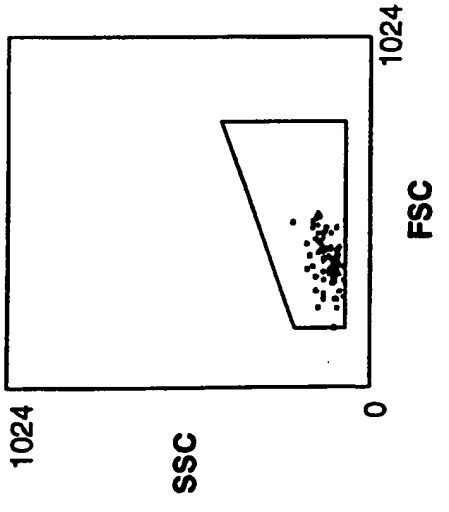


FIG - 6E

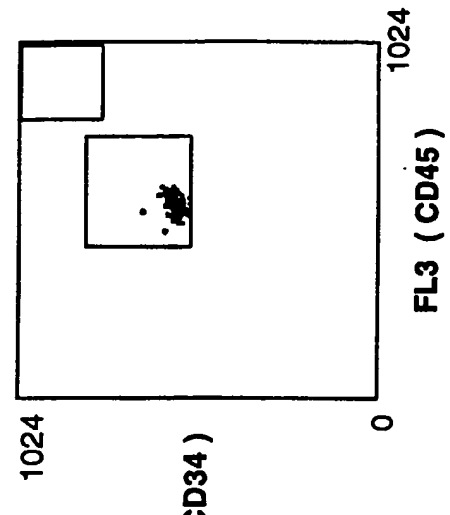


FIG - 6H

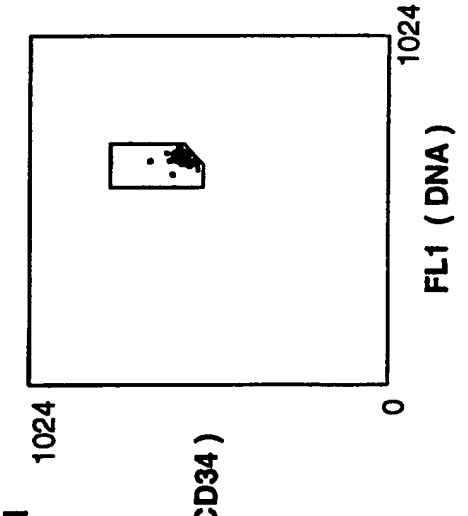


FIG - 6G

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12952

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/545, 33/546, 33/566
US CL :435/7.21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 250/459.1, 461.2; 435/7.21; 436/10, 43, 63, 172, 548, 800, 805

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,137,809 (Loken et al) 11 August 1992, see entire document.	1-5
Y	US, A, 5,047,321 (Loken et al) 10 September 1991, see entire document.	1-5
Y	US, A, 4,751,188 (Valet) 14 June 1988, see entire document.	1-5

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 JANUARY 1995	Date of mailing of the international search report 06 FEB 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Christopher Chin Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*